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Electron Spin Resonance Study of Rapidly Frozen Solution of Iron–Glutathione

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Abstract

Rapidly mixed anaerobic solutions (at pH 2.7) of $FeCl_3$ and glutathione were quickly frozen at various times after mixing. EPR spectra of these frozen solutions showed the progressive reduction of the iron(III) with time and the transient presence of a $g = 2$ radical signal. This signal is discussed in terms of an intermediate in the reduction pathway containing a high spin iron(II) centre weakly coupled to a sulphur radical

Similar experiments were carried out at pH 9 in the presence of oxygen.

Introduction

Previously we have reported studies on the reactions between ferric iron and glutathione (GSH) in aqueous solutions using Mössbauer spectroscopy and fast reaction kinetic methods [1–3]. At all pH values studied, GSH reduced Fe(III) to Fe(II) yielding oxidised glutathione [1–3]. We found no evidence for any long lived intermediates [2], (e.g. glutathione radicals) using NMR techniques (Evan's method).

A number of transient intermediates in the reaction pathway were identified optically and their Mössbauer spectra have been described [1]. In particular, in rapidly frozen solution, a pink intermediate was identified at low pH. The Mössbauer spectrum identified this as a high spin Fe(III) species, which is possibly coordinated to sulphur [1].

At high pH values (around 9.0), the reaction between an Fe(II)/GSH complex with molecular oxygen was found to proceed via a red Fe(III) intermediate [3].

In our previous publications [1, 3], we have postulated a series of mechanisms that involve GS radicals as the products of the initial Fe(III) reduction steps. As such radicals would be observed using electron paramagnetic resonance spectroscopy, the logical continuation of our work was such an investigation. The use of EPR in this study has the additional appeal that high-spin Fe(III) has an EPR signal whereas high-spin Fe(III) does not, thus providing a useful signal for monitoring the extent of the iron reduction. We report here EPR studies at pH 2.7 and pH 9.0 on the iron glutathione systems.

Experimental

Acid Solutions

Solutions of iron(III) chloride (or nitrate) were mixed in a standard EPR quartz tube with excess of GSH. The final pH was 2.7. The EPR tube was either immersed immediately, or after delay times between 5 and 20 s after mixing in isopentane cooled by liquid nitrogen. Freezing was complete in approximately 2 s [4].

Glutathione (reduced form) was purchased from Sigma Chemical Co. All chemicals were of analytical grade.

Alkaline Solution

Iron(III) chloride (10^{-3} M) was mixed anaerobically with a 10-fold excess of GSH. The resulting mixture was adjusted to pH 9.0 with 1 M NaOH. A small portion of the mixture was transferred anaerobically to an EPR tube using a syringe. The solution in the tube was equilibrated with air which made an intense red colour develop in a few seconds.

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While still red, the solution was then rapidly frozen in the cold isopentane bath.

EPR Spectroscopy

EPR spectra at 9 GHz were recorded on a Varian E-9 spectrometer equipped with an Oxford Instruments EPR-9 helium-flow cryostat or on a Varian E-3 spectrometer at 77 K.

Results and Discussion

Acid Intermediates

Rapidly frozen solutions (pink) containing iron(III) and GSH showed two major EPR signals at temperatures between 5 and 20 K (Fig. 1). These signals were centred around the g-values 4.3 and 2.

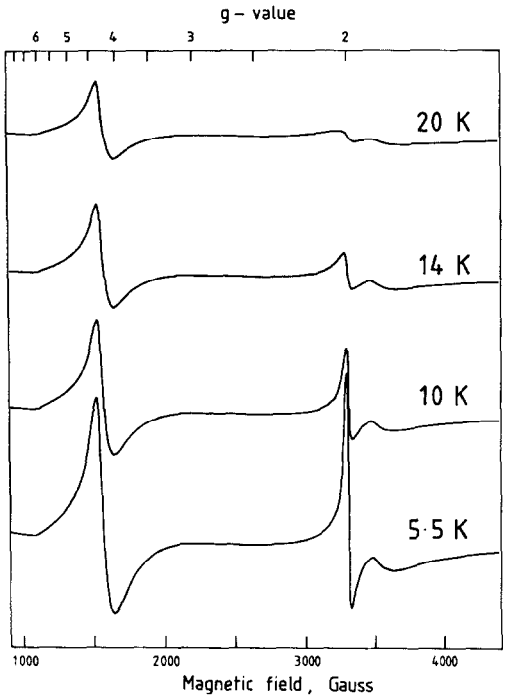

Fig. 1. EPR spectra of rapidly frozen solutions (pink) of $FeCl_3$/GSH in a 1:3 ratio at pH 2.7. [$FeCl_3$] = 10 mM. The samples were frozen immediately after mixing. The pure GSH solution, as well as the empty EPR cavity, generated only a straight line (no EPR signal). The conditions of the EPR spectroscopy were: microwave power, 2 mW; microwave frequency, 9.22 GHz; modulation amplitude, 2 mT; temperature, as shown in the Figure.

The signal at $g = 4.3$ is typical of high-spin iron(III) with $S = 5/2$, seen in non-haem iron complexes with very low symmetry (rhombic) [5, 6]. In the presence of GSH this signal disappears over a period of 20 s at the concentrations used in this work (see Fig. 2). We interpret this disappearance as evidence that the Fe(III) high-spin species is reduced by glutathione at this pH in agreement with data obtained using other techniques [1].

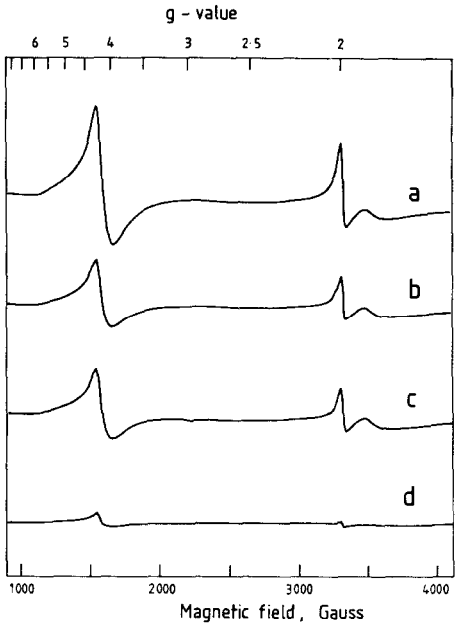

Fig. 2. EPR-spectra of a similar $FeCl_3$/GSH solution as in Fig. 1. The delay times between mixing and quench freezing were: (a) <1, (b) 5, (c) 10, (d) 20 s. EPR-conditions were as described in the legend to Fig. 1 except that the temperature was 10 K.

The signal around $g = 2$ is more complex and can be interpreted in at least two ways. Either (a) it is composed of at least two signals, one a narrow line (most likely due to a $S = ½$ radical) and the other a broad signal underlying the radical. The latter broad signal could be generated by an Fe environment e.g. 2Fe–2S (or more) as found in iron–sulphur proteins [5, 7, 8] where the individual g-values smear out; or (b) it could be accounted for by a model in which the $g = 2$ signal arises from an $S = 2$ system weakly coupled to an $S = ½$ system, giving a total $S = 3/2$ system. Such a system could be a high-spin Fe(II) coupled to a glutathione radical (GS).

The radical signal is seen to disappear during the course of the reduction of the iron (Fig. 2: 20 s) and is not present initially in solutions of FeCl or GSH. This behaviour is consistent with the presence of a transient radical intermediate in the reaction of iron(III) with GSH. The temperature dependence of the $g = 2$ signal (Fig. 1) shows that the species responsible for this signal has a short relaxation time. In addition (not shown) this signal was not saturated at power levels up to 80 mW at 10 K. This suggests the close proximity of the radical to a metal centre consistent with suggestion (b) above.

We have previously suggested from a rapid-kinetic study of the iron(III)–GSH reaction that the mechanism of reduction involves an iron(II)–GS inter-

mediate. The behaviour of the $g = 2$ species observed in the EPR experiments is consistent with this mechanism, indicating that an antiferromagnetically coupled system (Fe(II) weakly coupled to a GS radical) is a short lived intermediate in the GS–Fe(III) reaction.

This EPR evidence is also in keeping with the hypothesis [1] that the sulphur of the glutathione initially binds to Fe(III) and that following electron transfer it is the sulphur radical bound to Fe(II) that is observed in the EPR spectrum. The sulphur radical is then released from Fe(II) in a fast step and then reacts with a similar radical to form GSSG.

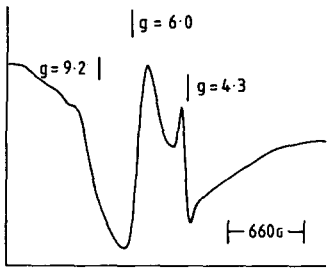

Fig. 3. EPR spectrum of a $FeCl_3$/GSH solution (red) in a 1:10 ratio at pH 9.0. [$FeCl_3$] = 1 mM. The sample was frozen immediately after equilibrating with oxygen. The conditions of EPR spectroscopy were: microwave power, 2mW; microwave frequency, 9.43 GHz; modulation amplitude, 2 mT; temperature, 15 K.

Red Intermediate

The EPR signals seen in the red intermediate (Fig. 3) are very similar to those seen in Fe(III) tetrathiolate anions [10]. Such anions give g-values at 8.4, 5.3 and 4.3 which was explained as stemming from highly asymmetric iron(III)$(SR)_4$ units. The $g = 4.3$ value is similar to that of rubredoxin [5], which contains Fe(III) bound to sulphur. This is consistent with our proposed mechanism [3] for the reaction of a Fe(II)–glutathione complex with oxygen. In this mechanism, it is the red intermediate iron(III)–sulphur complex that contains bound oxygen.

Upon thawing the red frozen material became colourless. After refreezing, no EPR signals could be found. This is indicative of iron(II) (the iron(III) having been re-reduced by excess glutathione); this is in agreement with the interpretation of the Mössbauer spectroscopic results [3].

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